

Immunospecificity of Albumin Detected in 1.6 Million-Year-Old Fossils From Venta Micena in Orce, Granada, Spain

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ABSTRACT The Orce skull fragment from southern Spain, dated at 1.6 Myr, has been a subject of heated controversy since it was first discovered in 1982. If it is hominid, as its discoverers contend, it is by far the oldest fossil hominid yet found in western Europe and implies that human populations settled this region much earlier than was previously realized. Numerous stone artifacts found at the Orce sites provide evidence that hominids were indeed present there in the Lower Pleistocene. Some paleontologists maintain that the 8 cm diameter occipital fragment is from a horse, not a hominid. Two independent investigations of the residual proteins in the skull were undertaken, one at the University of Granada in Spain, the other at the University of California, San Francisco. Two immunological methods of comparable sensitivity were employed for detection and species attribution of protein extracted from fossil bone: the Granada team used an enzyme-linked-immunosorbent assay (ELISA), and the UCSF team used a radioimmunoassay (RIA). Both teams obtained reactions characteristic of human albumin in the Orce skull and horse albumin in some of the horse fossils. These results support the lithic evidence that hominids were living in Andalusia 1.6 million years ago. *Am J Phys Anthropol* 103:433–441, 1997. © 1997 Wiley-Liss, Inc.

The fossil and archaeological open-air sites near Orce, in southern Spain, are situated around an ancient lake basin with a sequence of sediments continuous from the Middle Pliocene to the Middle Pleistocene. Chronology is based on paleomagnetism and micro- and macromammalian fauna. Situated between the Jaramillo event (0.9–1.05 Mya) and the Olduvai subchron (1.8 Mya) are three sites that suggest an early hominid presence. Fuentenueva 3 contains lithic cores, manuports, and small flakes. Barranco Leon (1.6–1.8 Mya) has yielded more than 60 artifacts. The lithic industry is characterized as similar to the Oldowan and Developed Oldowan in sub-Saharan Africa.

The Venta Micena site (1.6 Mya) contains a rich fossil-bearing layer from which 15,000 bones have thus far been unearthed, comprising more than 30 species. This bone accumulation resembles those around the dens of recent hyenas, and indeed bones of the giant hyenid *Pachycrocuta brevirostris* are abundantly represented (Gibert et al., 1992; Zihlman and Lowenstein, 1996).

From Venta Micena, three potentially hominid remains were identified by Gibert

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(Gibert et al., 1994a) and assigned to the genus *Homo* based on morphology (Campillo, 1992; Gibert et al., 1989; Gibert and Palmqvist, 1995): VM-0, a parietal-occipital fragment from a presumed infant or juvenile; VM1960, a juvenile humeral diaphysis; and VM3961, the mid shaft of an adult humerus. None of the three is sufficiently complete that species can be diagnosed with assurance. Some paleontologists maintain that VM-0 is from the skull of a horse rather than a hominid (Agustí and Moyá-Solá, 1987). Small pieces (50–100 mg) of these specimens, together with larger amounts from undisputed equid and bovid fossils, were provided to teams at the University of Granada in Spain and at the University of California, San Francisco for analysis. Neither team was aware of the other's activities, and there was no communication between them until after the final results of each group had been submitted to Gibert.

Fossil biomolecules may aid in family, genus, or species identification when the anatomical interpretations are disputed. All the characteristics of a species are programmed by its DNA, so the identification of nucleic acid sequences or their translated messages into proteins can in principle lead to accurate species identification. Morphology is also determined by DNA and proteins, but the connection involves many complicated steps and can be confused by convergent trends in unrelated species. Dozens of different genes may contribute to the shape of a tooth or a skull.

Fossil molecules of DNA or proteins, if they survive at all, are present in such low amounts that very sensitive techniques are necessary to detect them. Radioimmunoassay (RIA), which can measure nanograms (billionths of a gram) of protein, has been used to detect collagen and albumin in fossils such as a 1.9 My *Australopithecus robustus* (Lowenstein, 1981) and helped to resolve the disputed phylogenetic affinities of extinct mammoths, mastodons, Steller's sea cow, the Tasmanian wolf, and the quagga (Lowenstein and Scheuenstuhl, 1991). The ELISA method used by the Granada team has a sensitivity similar to that of RIA, is routinely used in medical diagnosis and research as a nonradioactive alternative to

RIA, and has also been used to study fossil proteins mostly from remains tens of thousands of years old. (Tuross and Stathoplos, 1993).

There is considerable controversy at present over the length of time that biomolecules are able to survive in fossil material. Lindahl (1993) has maintained on theoretical grounds that DNA will break down beyond recognition within a few thousand years. Nevertheless, a number of investigators have reported extracting DNA from insects preserved in amber, some as old as 30 Mya (DeSalle et al., 1993) and 120 Mya (Cano et al., 1993). Golenberg et al. (1990) have reported extraction of DNA from 17-My-old magnolia leaves found in a desiccated lake bed. Though none of these results has been accepted without challenge, it seems that under special circumstances, particularly the exclusion of water, informative biomolecules may survive for very long periods of time (Eglinton and Curry, 1991).

Proteins in fossil material also undergo diagenetic changes with time, including fragmentation, racemization, and conversion of amino acids into other amino acids (Hare et al., 1980). The advantage of an immunological approach is that most antibodies bind to sequences of six to ten amino acids (Benjamin et al., 1984), and these sequences may persist even after the proteins have deteriorated due to time, temperature, or chemical exposure (Lowenstein, 1993). We report here the finding of humanlike immunological reactions in 1.6-million-year-old fossils from Orce.

MATERIALS AND METHODS

Fossil extracts

Table 1 summarizes the characteristics of the fossils we studied. Fresh human bone (a fragment of diaphysis of femur) obtained from a surgical procedure was also included for comparison. The sample of fossil was ground to a fine powder and decalcified in a solution of 0.2 M EDTA, pH 7.4, for 1 week in continuous agitation at room temperature. The extract was centrifuged, and the supernatant was collected, divided, and stored at -70°C until use. Fossils VM-0 and VM1960 were washed in phosphate-buffered saline (PBS) for 1 week before they were decalcified

TABLE 1. List of fossils studied in this work

Fossil	Assigned to	Anatomical location	Age	Found in
VM-0	Hominid	Skull fragment	1.6 Myrs	Venta Micena, Orce
VM1960	Hominid	Humerus fragment	1.6 Myrs	Venta Micena, Orce
CV-1	Hominid	Humerus fragment	1.4 Myrs	Cueva Victoria, Murcia
EEG	Equid	Skull fragment	1.6 Myrs	Venta Micena
VM3575	Equid	Mandible fragment	1.6 Myrs	Venta Micena, Orce
VM64	Equid		1.6 Myrs	Venta Micena
VM1653	Equid		1.6 Myrs	Venta Micena
CV-5	Equid		1.4 Myrs	Cueva Victoria
VM786	Bovid		1.6 Myrs	Venta Micena
VM1587	Bovid		1.6 Myrs	Venta Micena

by EDTA. PBS supernatant was also collected, divided, and stored. Contamination of fossil material with molecules from human skin and hair is a recognized difficulty in these analyses. To minimize this risk during the process of extraction and testing, workers wore gloves and a face mask while working with the samples, and extreme care was taken to use clean or disposable materials. All samples were tested blindly; that is, the identity of the samples was unknown when they underwent laboratory analyses.

Antibodies

For the ELISA method, polyspecific mouse anti-human albumin and anti-horse albumin sera were obtained by intramuscular injection of BALB/c mice with the protein emulsified in Freund's complete adjuvant. Mouse monoclonal antibodies (mAbs) to human albumin were also employed: MEGA-1, MEGA-2, and MEGA-3. All three monoclonal antibodies cross-react with baboon albumin but not with horse albumin (Garcia-Pacheco, 1990).

For the radioimmunoassay (RIA) method, polyspecific rabbit anti-human, anti-horse, anti-bison, and anti-bear albumin sera were obtained by intramuscular injection of New Zealand rabbits with the protein emulsified in Freund's complete adjuvant, followed by three booster injections without adjuvant.

Detection of albumin by ELISA

At the University of Granada, Spain, albumin in fossils was detected with a conventional ELISA with avidin-biotin complex (Sigma, St Louis, MO). One hundred microliters of the fossil extract or of a solution of recent human, baboon, or horse albumin (Sigma) was placed in the wells of a microti-

ter polystyrene plate (Nunc, Austria), and proteins were left to attach to the plastic for 3 h at 37°C. During this process, some proteins became irreversibly attached to the plastic. Any plastic binding sites in the plates which remained free were blocked with 1% gelatin for 1 h at room temperature. Then appropriate mouse serum or monoclonal antibody to specific albumin was added, and the plates were incubated for 1 h at room temperature. The second antibody used was a biotinylated goat anti-mouse IgG (Sigma). After 2 h of incubation at 37°C, extravidin-peroxidase (Sigma) was added, and the plates were again incubated for 30 min at room temperature. After each step the plates were washed with PBS containing 0.05% Tween-20. The enzymatic reaction for peroxidase was carried out with o-phenylene diamine (OPD) and H₂O₂. The plates were read at 490 nm with a microplate autoreader. Absorbances obtained with fossil extracts were compared with the calibration curves of fresh human, baboon, and horse albumin in order to quantify the albumin detected (Fig. 1).

Detection of Albumin by Radioimmunoassay (RIA)

At the University of California, San Francisco, fossil extracts or standard sera (20 µl) were placed in the wells of polyvinyl microtiter plates (Dynatech, Chantilly, VA) and allowed to remain for 2 h at room temperature, during which time protein bound irreversibly to the plastic. The wells were then washed with a solution containing soy protein to block any additional binding sites. Rabbit antisera to various species of albumins were added to the wells for 24 h and washed out, and then a second antibody,

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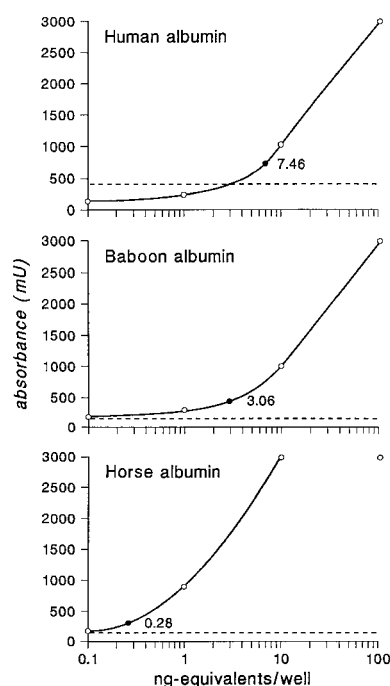


Fig. 1. Reactivity with an EDTA extract of VM-0 (3 mg bone/ml) of three mouse polyspecific sera against human, baboon, and horse albumin. Note that VM-0 gives the strongest reaction (7.46) with antiserum to human albumin, a moderate reaction (3.06) with anti-baboon albumin, and almost no reaction (0.28) with anti-horse albumin. Reactivities with the fossil extract, obtained as mU absorbance, were extrapolated on the corresponding calibration curve (○—○) to obtain values, in nanogram equivalent/well, of albumin of each species. Numbers near the fossil reactivity mark (●) represent the nanogram equivalents/well of albumin obtained after extrapolation. Dotted line represents the background.

I-125-labeled goat antirabbit gamma globulin (GARGG), was added for 24 h and washed out. Radioactive counts in the individual cups were then measured in a scintillation counter and compared to known amounts of albumin in standard human, horse, bison, and bear sera.

Relative immunological similarity

In the ELISA technique, fossils were tested with antisera against albumin from three different species: human, baboon, and horse. The amount of species-specific albumin in each fossil was estimated by comparing the

fossil reactions with homologous antigen-antibody reactions (Fig. 1). Relative immunological similarity (RIS) is defined as the ratio of each reaction to the homologous (most specific) albumin determination. The highest RIS, indicating identity of the tested and the homologous reaction, is 1. The weakest RIS, indicating no specific reaction, is 0.

In the RIA technique, the results were quantitated in two ways: by the absolute amount of the radioactive second antibody bound to the cup (Table 3) and by the amount of albumin (in nanograms per milliliter) obtained from comparison with weak dilutions of human, horse, bison, and bear serum (Table 4).

RESULTS

ELISA method

Detection of immunospecific albumin in fossil bones. Figure 1 shows the reactions of an extract of fossil VM-0 with three antisera against human, baboon, and horse albumin. The highest value was obtained with the antiserum against human albumin, followed by baboon albumin, and the lowest was with horse albumin. These results indicated that the albumin found in VM-0 was immunologically closer to man than to the other two species. Similar results were found with VM1960. Human albumin from fresh bone also reacts more strongly with anti-baboon than with anti-horse antiserum, as would be expected, though quantitative differences in the ratios suggest that the fossil proteins have undergone significant structural changes. In contrast, the albumin detected in the equid fossil EEG was immunologically closer to horse albumin than to human or baboon albumin (Fig. 2). We did not detect albumin in fossils CV-1 and VM3575 (Table 2) or in the solutions used for the fossil extractions (not shown).

We did not detect any discernible pattern as to which bone gave the best results.

PBS extracts of fossils VM-0 and VM1960.

Fossils VM-0 and VM1960 were maintained in phosphate-buffered saline (PBS) for 1 week in continuous agitation (see Materials and Methods). Any albumin contaminating the surface of the bones would have gone

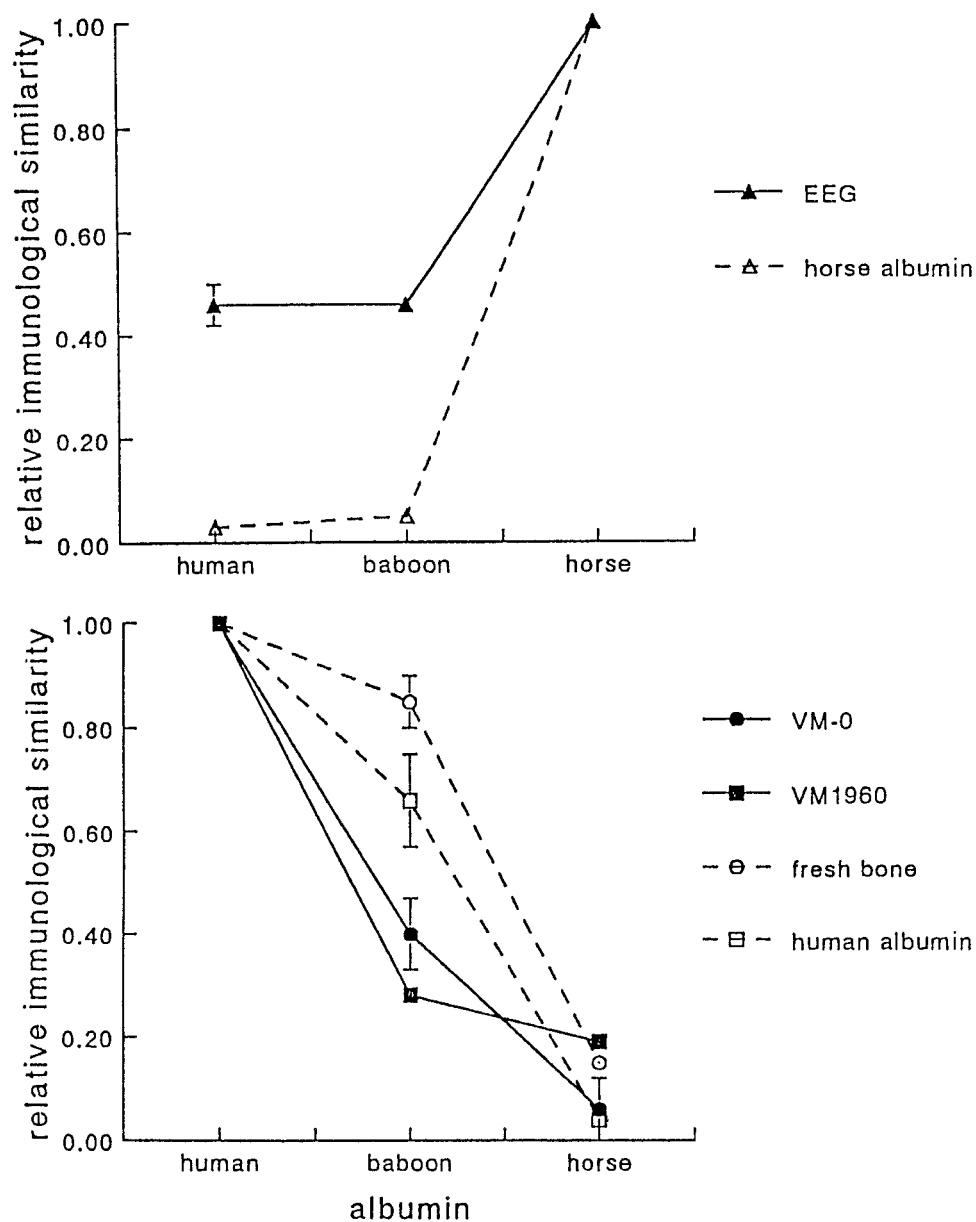


Fig. 2. Fossil albumin is compared to fresh human, baboon, and horse albumin. Relative immunological similarity of the albumin detected in the EDTA extracts of VM-0 (results are means of three independent extractions, 1, 3, and 5 mg/ml), VM1960 (3.5 mg/ml), EEG (2.8 mg/ml), and fresh human bone (5 mg/ml) to human, baboon, and horse albumin. Ten nanograms/well of human and horse albumin were used as controls. VM-0 and VM1960 (putative hominids) give a pattern of reactions similar to human albumin, while EEG (fossil horse) gives a pattern of reactions similar to horse albumin.

into solution in PBS. No albumin was detected in the PBS extracts of these fossils. This protein was detected only when the fossils were decalcified by treatment with

EDTA, suggesting that the material was deeply integrated in the fossil structure. Furthermore, this protein was not found in soil collected near VM-0 (Table 2).

TABLE 2. ELISA method: Reactivity of polyspecific mouse sera against human and horse albumin and of mouse anti-human albumin monoclonal antibodies (MEGA-1, MEGA-2, and MEGA-3) with fossils extracts

Antibody	Fossil	Extracted in	Albumin (ng)/bone (mg)
Mouse serum anti-human albumin	VM-0	PBS (5 mg/ml) ¹	0.0 ± 0.0
Mouse serum anti-human albumin	VM-0	EDTA (5 mg/ml)	9.8 ± 0.5
Mouse serum anti-human albumin	VM-0	EDTA (3 mg/ml)	20.7 ± 1.2
Mouse serum anti-human albumin	VM-0	EDTA (1 mg/ml)	25.7 ± 0.2
MEGA-1	VM-0	EDTA (3 mg/ml)	48.0 ± 10.2
MEGA-2	VM-0	EDTA (3 mg/ml)	1.4 ± 0.3
MEGA-3	VM-0	EDTA (1 mg/ml)	39.1 ± 10.1
Mouse serum anti-human albumin	Soil collected near VM-0	EDTA (21 mg/ml)	0.0 ± 0.0
Mouse serum anti-human albumin	VM1960	PBS (3.5 mg/ml)	0.0 ± 0.0
Mouse serum anti-human albumin	VM1960	EDTA (3.5 mg/ml)	14.5 ± 0.8
Mouse serum anti-human albumin	VM1960	EDTA (1 mg/ml)	14.7 ± 7.7
MEGA-1	VM1960	EDTA (1 mg/ml)	155.1 ± 32.2
MEGA-2	VM1960	EDTA (1 mg/ml)	7.8 ± 0.0
MEGA-3	VM1960	EDTA (1 mg/ml)	8.1 ± 0.4
Mouse serum anti-human albumin	CV-1	EDTA (3.5 mg/ml)	0.0 ± 0.0
Mouse serum anti-horse albumin	EEG	EDTA (3 mg/ml)	5.3 ± 0.4
MEGA-1	EEG	EDTA (2.8 mg/ml)	0.0 ± 0.0
MEGA-2	EEG	EDTA (2.8 mg/ml)	0.0 ± 0.0
MEGA-3	EEG	EDTA (2.8 mg/ml)	0.0 ± 0.0
Mouse serum anti-horse albumin	VM 3575	EDTA (3.3 mg/ml)	0.0 ± 0.0

¹ Milligrams of fossil per milliliter of extraction solution.

TABLE 3. Radioimmunoassay (RIA) method

Antiserum	Fossil hominid VM-0 protein reactions, as % of bound antibody		
	Human	Horse	Bovine
Albumin	2.8	0.5	0.2
Serum	1.8	0.3	—
Transferrin	1.4	—	0.5
Collagen	0.7	—	0.2
Means	1.9	0.4	0.3

Reactions of monoclonal antibodies against human albumin with fossil extracts. All three monoclonal antibodies (mAbs) reacted with the fossil extracts from VM-0 and VM1960 but were negative with the equid fossil EEG. Albumin was, however, detected in this equid fossil with an antiserum against horse albumin (Table 2). The three monoclonal antibodies are known to react strongly with human albumin, cross-react somewhat with albumin from other primates, and react very weakly or not at all with albumin from nonprimate mammals (García-Pacheco et al., 1990). Thus, extracts from fossils VM-0 and VM1960 reacted like human albumin, while fossil EEG reacted like horse albumin.

RIA method

The results are shown in tabular form (Tables 3, 4). Fossil VM-0 reacted more

TABLE 4. RIA method: Albumin concentrations, nanograms/milliliters equivalent, in fossil extracts

	Anti albumins			
	Human	Horse	Bison	Bear
Hominid				
VM-0	4	0	0	0
VM1960	4	0	1	0
Equid				
VM64	0	0	0	0
VM1653	4	11	7	2
CV5	1	6	5	1
Bovid				
VM786	2	3	12	2
VM1577	2	4	28	2

strongly with antisera to human albumin, whole serum, transferrin, and collagen than it did with antisera to horse albumin and whole serum or to bovine albumin, transferrin, and collagen. Putative human fossils VM-0 and VM1960 reacted most strongly with anti-human albumin, equid fossils VM1653 and CV5 most strongly with anti-horse albumin, and bovid fossils VM786 and VM1577 most strongly with anti-bison albumin. None of the specimens reacted significantly with anti-bear albumin.

Table 5 combines and summarizes the ELISA and RIA results. The putative human fossils gave humanlike reactions, the equid fossils gave horselike reactions, the bovid fossils gave bisonlike reactions, and none of these gave bearlike reactions.

TABLE 5. Summary of ELISA and RIA reactions on Orce fossils¹

	Antisera to various albumin species					
	Anti-human			Anti-horse		Anti-bison Rp
	Mm	Mp	Rp	Mp	Rp	
Human fossils						
VM-0	30	19	4		0	0
VM1960	57	15	4		0	0
Horse fossils						
EEG		0		5		
VM1653			4		11	7
CV5			1		6	5
Bovine fossils						
VM1653			2		3	12
VM1577			2		4	28

Homologous reactions (human vs. human(?), horse vs. horse, bison vs. bovine) are shown in bold type.
Mm, mouse monoclonal; Mp, mouse polyclonal; Rp, rabbit polyclonal.

DISCUSSION

Our results demonstrate that the albumin detected in VM-0 is much closer immunologically to human than to horse albumin (Fig. 1, 2; Tables 2–5). Whole serum, transferrin, and collagen reactions also suggest that VM-0 is human (Table 3). Since paleontologists disagree whether the fossil is hominid or equid (Agustí and Moyá-Solá, 1987; Gibert et al., 1989), the immunological results are significant in supporting the contention that it is hominid. We obtained similar results with VM1960 (Fig. 2; Tables 2, 3), a humeral fragment found in the same bed as VM-0 and also attributed to a hominid (Gibert et al., 1994b). On the other hand, the albumin detected in undisputed equid fossils EEG, VM1653, and CV-5 was closer to horse than to baboon or human albumin (Fig. 2; Table 2) or to bison or bear (Table 3). No albumin reactions were obtained in CV-1, a putative hominid (Gibert et al., 1992), or in equids VM3575 and VM64 (Tables 2, 3).

A major issue in the study of biomolecules in fossils is the reliable exclusion of exogenous contamination. Because albumin is a highly soluble protein, fossils might be impregnated with perspiration or saliva during handling. VM-0 and VM1960 were tested for possible surface contamination by soaking them in a saline buffer, PBS, instead of the decalcifying solution EDTA. Surface contamination from human handling would be expected to show up in the saline wash. No immunoreactivity was observed in this solution, however, which indicates that the bone surface was free of albumin. The EDTA

solution dissolves bone calcium and releases protein that was integrated in the mineral structure of the fossils. Positive reactions in the EDTA extract therefore seem to indicate the persistence of endogenous albumin in the fossils. As a further control on external contamination, we analyzed soil collected within a circle 20 cm diameter around the spot where the Orce skull was found, and no reactivity was detected (Table 2). It is therefore unlikely that the fossils had been contaminated by human or animal remains in the soil. Though contamination of specimens with human DNA and proteins is a known hazard in the search for ancient biomolecules, the species-specific reactions on equine and bovine bones cannot be explained by contamination and suggest the persistence of molecular albumin or albumin fragments in the bony matrix.

The apparent amounts of albumin found in VM-0 and VM1960 varied markedly depending on the antibody used. Because fossil proteins may be denatured and broken up as a result of physical and chemical changes over time, the different reactivities of the monoclonal antibodies with fossil extracts probably reflect different states of preservation of the molecular fragments recognized (Lowenstein, 1981; Lowenstein and Scheuenstuhl, 1991). Extensive studies carried out on albumin immunology have shown that, when albumin is injected into animals such as mice or rabbits, the animals produce many different antibody clones (polyclonal antisera) to different parts of the albumin molecule (Benjamin et al., 1984). As long as

some of the original configuration remains in the altered molecule, some antibodies will continue to bind to it. Monoclonal antibodies, however, will react with only a single short sequence or configuration in the large (590 amino acid) albumin molecule, and this particular sequence may or may not persist in the fossil fragments. It would be expected that three different monoclonal antibodies, directed against three different regions, would show unequal reactivity, depending on the degree of survival of specific regions of the albumin molecule. Our results do show such variable reactivity (Table 2). Monoclonal antibodies may eventually provide us with important data about rates at which particular portions of the albumin molecule break down over time under different environmental conditions.

Poinar et al. (1996) showed that DNA does not survive in fossils in which racemization of the amino acid aspartic acid exceeds a D/L ratio of 0.08. This means "that the survival of DNA is limited to a few thousand years in warm regions such as Egypt and to roughly 100,000 years in cold regions." However, Poinar et al. (1996) also report that amino acids, the constituents of proteins, are surprisingly well preserved in amber-entombed insects and speculate that this may be due to the exclusion of water. The persistence of amino acids for millions of years, even in fossils devoid of DNA, suggests that protein residues may also survive in some of these materials. Tomas Lindahl, whose 1993 article is widely quoted by those who question the prolonged survival of ancient DNA, stated in a personal communication with one of the present authors (J.M.L.) that on biochemical grounds he thinks proteins are more likely to persist than DNA.

We do not know why proteins have been so exceptionally well preserved at the Orce site, especially at Venta Micena, which appears to be an accumulation around hyena dens, in an ancient lake bed. The bones here have a chalky white appearance that may reflect an alkaline condition, in contrast with the usual acid state of soil that accelerates hydrolysis of both DNA and proteins.

Immunological albumin reactions in fossils about 1.6 Myr old, although surprising, are not entirely improbable, in the light of

the observation with electron microscopy of collagen fibrils in a 200-Myr-old dinosaur bone (Wykoff, 1972) and immunological reactions in 70-Myr-old molluscs (De Jong et al., 1974); osteocalcin has been extracted from 13-Myr-old fossil bovid bones and from 30-Myr-old fossil rodent teeth (Ulrich et al., 1987); collagen has been reported in a 10-Myr-old unidentified bone (Rowley et al., 1986); and species-specific serum factors have been detected in a 0.5-Myr-old *Homo erectus* fossil (Lowenstein, 1981) and in 8-Myr-old *Ramapithecus* and *Sivapithecus* specimens (Lowenstein, 1983).

Due to its faster rate of evolution (Wilson et al., 1977), albumin discriminates between species much better than collagen and osteocalcin. Albumin has previously been used to resolve controversies about the phylogenetic status of several extinct species. To our knowledge, this is the first time albumin has been detected and used for generic identification in such ancient fossils.

These results have important implications for the study of hominid evolution. Debates about morphological identification of fragmentary fossils cannot be resolved when equally qualified experts contend that the same specimen is either a hominid or a horse. In the case of the partial skull VM-0, two independent research teams have found humanlike albumin within the bone. This finding supports the evidence of the many lithic tools unearthed at the Orce sites indicating that hominids inhabited Europe, and specifically southern Spain, at a much earlier date than was previously recognized.

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